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אני, (שם המבקש, מענו ולגבי גוף מאוגד - מקום התאגדותו)
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ידע חברה למחקר ופיתוח בע"מ, חברה ישראלית, ליד מכון ויצמן למדע, ת.ד. 95, רחובות 76100
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Hematopoietic Cell Composition for Use in Transplantation

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HEMATOPOIETIC CELL COMPOSITION FOR USE IN TRANSPLANTATION

תכשיר המכיל תאים המטופויטיים לשימוש בהשתלות

Yeda Research and Development Co. Ltd.
Inventors: Tsvee Lapidot and Amnon Peled

ידע חברה למחקר ופתוח בע"מ
ממציאים: צבי לפידות ואמנון פלד

FIELD OF THE INVENTION

The present invention relates to compositions comprising hematopoietic cells and their use in transplantation.

BACKGROUND OF THE INVENTION

Hematopoietic stem cells are rare cells within the bone marrow population that actively maintain continuous production of all mature blood cells throughout the entire life span (Morrison et al., 1995; Ogawa, 1993). Stem cells are defined based on their functional ability to home to the bone marrow and to durably repopulate transplanted recipients with both myeloid and lymphoid cells. Much of our knowledge of the regulation and the hierarchical organization of the hematopoietic system derives from studies in the mouse wherein stem cells are identified and quantified in long-term reconstitution assays (Morrison and Weissman, 1994; Spangrude et al., 1991). In contrast, our knowledge of the biology of human hematopoiesis is limited, since it is mostly based on in vitro assays, or clinical bone marrow transplantation protocols, both lacking the option to characterize and quantify repopulating stem cells (Sutherland et al., 1995; To et al., 1997).

Recently, several groups have established in-vivo models for normal and leukemic human hematopoiesis by implantation or transplantation of primitive cells into immune deficient mice, or in utero into fetal lambs. Injection of immature cells into human fetal tissues previously implanted into SCID mice, transplantation into irradiated Beige Nude Xid, SCID and NOD-SCID mice, and in utero transplantation into sheep fetuses, all resulted in successful multi-lineage engraftment of both myeloid and lymphoid cells (McCune et al., 1988; Nolta et al., 1994; Lapidot et al., 1992; Larochelle et al., 1996; Civin et al., 1996). Previously we developed a functional in-vivo assay for primitive human hematopoietic cells based on their ability to durably repopulate the bone marrow of intravenously transplanted SCID and more recently NOD/SCID mice with high levels of both myeloid and lymphoid cells. We defined the engrafting cell as the SCID Repopulating Cell (SRC) (Lapidot et al., 1992; Larochelle et al., 1996; Dick et al., 1997). Kinetic experiments demonstrated that only a small fraction of

the transplanted cells engrafted and that these cells repopulated the murine bone marrow by extensive proliferation and differentiation (Lapidot et al., 1992; Cashman et al., 1997). Furthermore, the primitive human cells also retained the capacity to engraft secondary murine recipients (Cashman et al., 1997). Transplantation of populations enriched for CD34 and CD38 cell surface antigen expression, revealed that the phenotype of SRC is $CD34^{+}/CD38^{-}$ (Larochelle et al., 1996; Dick et al., 1997). Other repopulating cells may exist since recent studies suggest that immature human CD34 negative cells and more differentiated $CD34^{+}/CD38^{+}$ cells have some limited engraftment potential (Zanjani et al., 1998; Civin et al., 1996; Conneally et al., 1997).

Accumulating evidence indicates that stem cell homing to the bone marrow is a multi-step process. The mechanisms and specific adhesion molecules involved in this process are not fully understood. The β1 integrins, very late antigen 4 (VLA-4), VLA-5 and the β2 integrin lymphocyte function-associated 1 (LFA-1) have been implicated in the adhesive interactions of both mouse and human progenitor cells with the bone marrow extracellular matrix, as well as with bone marrow stromal cells (Levesque et al., 1995; Teixido and Anklesaria, 1992). VLA-4 plays an especially important role in murine stem cell migration and hematopoiesis in-vivo. Murine stem cells lacking β1 integrins fail to colonize the fetal liver (Hirsh et al., 1996). Similarly, homing of lymphocytes into lymphoid tissue and migration of leukocytes to inflammation sites are also mediated by adhesion molecules and by an entire family of chemoattractant cytokines (chemokines) and their cell surface receptors (Ahuja et al., 1994; Premack and Schall, 1996; Anderson et al., 1996). Chemokines, a large family of cytokines that are characterized as signaling molecules, are best known for their ability to selectively attract subsets of leukocytes to inflammation sites. However, chemokines are also important regulators of human development, hematopoiesis and angiogenesis (Horuk and Peiper, 1996; Premack and Schall, 1996). Activation of chemokine receptors in leukocytes results in a multi step process that includes activation of cell surface adhesion molecules followed by firm adhesion to the vessel wall and eventually migration into the extravascular compartment (Premack and Schall, 1996). A noted chemokine is stromal cell-derived factor-1 (SDF-1), also known as Pre-B cell growth stimulating factor (Nagasawa et al., 1996). Human and murine forms of SDF-1 differ in one amino acid and are cross reactive. SDF-1, is the ligand for the chemokine receptor CXCR4 (fusin, LESTR), which is

expressed on human lymphoid, myeloid and $CD34^+$ cells (Bleul et al., 1996; Oberlin et al., 1996; Bautz et al., 1997; Deichmann et al., 1997). SDF-1 is a powerful chemoatractant for $CD4^+$ T cells as well as for $CD34^+$ cells, and was shown to induce rapid activation of LFA-1 and VLA-4 on human $CD4^+$ T cells (Bleul et al., 1996; Aiuti et al., 1997; Kim and Broxmeyer, 1998; Mohle et al., 1998; Campbell et al., 1998). Interestingly, mice that lacked SDF-1 or CXCR4 expression exhibited a severe defect of both lymphoid and myeloid hematopoiesis in the fetal bone marrow (Nagasawa et al., 1996; Nagasawa, 1998; Zou et al., 1998). A defect in stem cell homing to the bone marrow, may be one explanation for such a phenotype. CXCR4 has also been shown to function as the major coreceptor for HIV on human T cells (Bleul et al., 1996; Oberlin et al., 1996). $CD34^+$ cells are also prone for HIV infection via their CXCR-4 receptor (Carr et al., 1998; Deichmann et al., 1997).

SUMMARY OF THE INVENTION

The processes that mediate homing and engraftment of human stem cells to the bone marrow involve a complex interplay between cytokines, chemokines and adhesion molecules, though details of this regulatory system are poorly understood.

It has been found, according to the present invention, that the chemokine SDF-1 and its receptor CXCR4 are essential for murine bone marrow engraftment by immature human $CD34^+$ cells; that in-vitro, SDF-1 mediates chemotactic migration and integrin activation leading to firm adhesion to endothelial ligands, and that the function of SDF-1 is regulated by stem cell factor (SCF). This cytokine induced surface expression of CXCR4 on $CD34^+$ cells and potentiated migration and engraftment. The latter processes apparently depend on the major integrins LFA-1, VLA-4 and VLA-5, since antibodies to these integrins interfered with migration and engraftment.

We thus identified immature human $CXCR4^+$ cells endowed with migration and bone marrow repopulation potential. These findings delineate key steps in the complex engraftment process and suggest upregulation of CXCR4 as a novel approach to expand stem cells for clinical transplantation.

While studying the mechanism by which the chemokine SDF-1 and its receptor CXCR4 regulate migration and engraftment of primitive human SCID repopulating cells, it was found according to the present invention that a chemotactic gradient of SDF-1 mediates in

vivo migration and engraftment of human CD34+/CXCR4+ cells to the bone marrow of immunodefficient NOD/SCID or NOD/SCID β2 microglobulin knock out mice, and that the cytokine SCF plays an important regulating role in these processes.

The present invention relates to a cell composition consisting essentially of a cellular population of viable mammalian immature hematopoietic cells including stem cells, which cells have the following characteristics:

(i) the cells are CXCR4+, namely the cells express or have the potential to express CXCR4, the cell surface receptor of the chemokine stromal-derived factor 1 (SDF-1);

(ii) the cells migrate in response to SDF-1;

(iii) the migration to SDF-1 through extracellular matrix is dependent on VLA-4 and VLA-5;

(iv) in response to SDF-1 the cells can activate LFA-1; and

(v) the cells can be successfully engrafted in a mammal, in which mammal they are capable of proliferation and differentiation into myeloid and/or lymphoid cells;

The cell population may be human cells from live donors or cadavers or are derived from any suitable non-human mammal such as pig, monkey, etc. In one preferred embodiment, the human cells are autologous or allogeneic cells.

The human immature hematopoietic cells may be derived from bone marrow, cord blood, fetal liver, yolk sac or mobilized peripheral blood. Mobilization of peripheral blood leukocytes (PBL) may be carried out by leukapheresis by stimulation of the donor with cytokines such as G-CSF, GM-CSF or mixtures of G-CSF+SCF and GM-CSF+SCF by techniques known in the art.

The immature hematopoietic cells including stem cells with the potential to express CXCR4, upon stimulation with an agent, express surface CXCR4 in a time-dependent manner. Examples of such agents that upregulate surface expression of CXCR4 are, without being limited to, lectins such as phytohemagglutinin (PHA) and cytokines involved in maintenance, expansion and/or development of stem cells such as SCF, IL-1, IL-6, IL-11 and GM-CSF, or a cocktail of cytokines such as SCF + IL-6 and SCF + GM-CSF. The cocktails of cytokines resulted in synergistic, higher levels of CXCR4 surface expression and constitute a preferred embodiment of the invention.

In another preferred embodiment according to the invention, the population of immature hematopoicells including stem cells that express or have the potential to express CXCR4 are stimulated with stem cell factor (SCF) for a period of time of 12 to 120 hours, preferably up to three days, most preferably for 36-48 hours.

The human cell composition of the invention may consist of human hematopoietic CD34- or CD34+ cells, both showing migration capability in response to SDF-1. In one preferred embodiment of the invention, the cells are CD34+ CXCR4+ cells.

CD34+ cells consist of 95-99% of CD34+/CD38+/high subpopulation and 1-5% of CD34+/CD38-/low cells. These latter cells have stem cell properties and are better for engraftment. Thus, in another preferred embodiment of the invention, the cell composition consists of human CD34+/CD38-/low/ CXCR4+ cells.

In another aspect, there is provided a method for preparing a cell composition according to the invention, comprising:

(i) removing red blood cells and granulocytes from bone marrow, cord blood, fetal liver, yolk sac or mobilized peripheral blood cells;

(ii) treating the cells from (i) to remove mature cells;

(iii) treating the thus obtained enriched population of immature cells from (ii) being CXCR4+ cells and cells with the potential to express surface CXCR4 with an agent for a determined period of time to enhance surface CXCR4 expression,

thus obtaining a cell composition consisting of an enriched population of mammalian immature hematopoietic CXCR4+ cells that include stem cells.

In one embodiment, the method for preparing a cell composition consisting of human hematopoietic CD34+ CXCR4+ cells according to the invention, comprises:

(i) removing red blood cells and granulocytes from bone marrow, cord blood, fetal liver, yolk sac or mobilized peripheral blood cells;

(ii) treating the cells from (i) with anti-CD34 monoclonal antibodies to enrich for population of CD34+ cells; and

(iii) treating the thus obtained enriched population of CD34+ cells from (ii) with an agent for a determined period of time to enhance surface CXCR4 expression,

thus obtaining a cell composition consisting of an enriched population of CD34+CXCR+ cells.

In these methods, removal of red blood cells, granulocytes and mature cells are carried out by techniques well-known in the art. The agent that upregulates surface expression of CXCR4 is as described above, but preferably the cells are treated with a cocktail of cytokines as described above or with SCF for up to three days.

The thus obtained enriched human hematopoietic CD34+CXCR4+ cell population may be further enriched to human hematopoietic CD34+/CD38-/low CXCR4+ cells, for example with anti-CD38 monoclonal antibodies.

In another aspect of the invention, there is provided a chimeric mammal transplanted with a cell composition of the invention, said chimeric mammal being capable of supporting the proliferation and differentiation of the transplanted immature hematopoietic cells including stem cells into myeloid and/or lymphoid cells.

In one preferred embodiment, the chimeric mammal is transplanted with a cell composition consisting of human hematopoietic CD34+CXCR4+ cells. In another preferred embodiment, the transplanted cells consist of human hematopoietic CD34+/CD38-/low / CXCR4+ cells.

The engraftment of the cells in the chimeric mammal is carried out by a process comprising:

(i) sublethally irradiating an immunodefficient mammal lacking a population of functional B and T cells; and

(ii) transplanting into the irradiated immunodefficient mammal the hematopoietic cell composition of the invention, optionally after treatment with an agent for a determined period of time to enhance cell CXCR4 expression.

The engrafted cells are preferably human hematopoietic CD34+CXCR4+ or CD34+/CD38-/low / CXCR4+ cells, optionally after treatment with an agent, e.g. SCF, for a determined period of time, e.g. 12 to 120 hours, to enhance surface CXCR4 expression.

The immunodefficient mammal may be a mouse, more particularly a NOD/SCID or a NOD/SCID β2-microglobulin-knock out (NOD/SCID β2M KO) mouse.

The chimeric mammal serves as a model for testing the engraftment efficiency of human immature hematopoietic CXCR4+ cells derived from bone marrow, cord blood, fetal liver, yolk sac or mobilized peripheral blood, by testing the level of human myeloid and

lymphoid cells in the mammal after engraftment of the human immature hematopoietic CXCR4+ cells tested.

The invention further provides an in vitro method for screening hematopoietic cells as candidates for transplantation into human hosts by testing the migration efficiency of hematopoietic CXCR4+ cells derived from bone marrow, cord blood, fetal liver, yolk sac or mobilized peripheral blood, said method comprising:

(i) measuring the level of cell surface CXCR4 expression in said cells with labeled anti-CXCR4 monoclonal antibodies; and

(ii) measuring the cells' ability to migrate in response to SDF-1 in a cell migration assay with a gradient of SDF-1, optionally across stromal cells or a stromal cell line,

the cells with a high migratory capability in response to SDF-1 being suitable for successful transplantation into human hosts.

In another aspect, the invention provides a method for testing and ascertaining the engraftment capability of human hematopoietic CXCR4+ cells derived from bone marrow, cord blood, fetal liver, yolk sac or mobilized peripheral blood, which cells have a high expression level of CXCR4 and high migratory capability in response to SDF-1, said method comprising transplanting said cells into a chimeric mammal of the invention as described above, whereby stable engraftment in the model chimeric mammal capable of supporting the proliferation and differentiation of said transplanted cells into myeloid and/or lymphoid cells, indicate the suitability of said cells for successful engraftment in human hosts.

In still another aspect, the invention provides a method for transplantation of immature hematopoietic stem cells in a patient in need therefor, said method comprising:

(i) conditioning the patient under sublethal, lethal or supralethal conditions; and

(ii) transplanting the conditioned patient with a cell composition of the invention.comprising human immature hematopoietic cells such as cells derived from bone marrow, cord blood, fetal liver, yolk sac or mobilized peripheral blood.

The human cells to be transplanted may be autologous or allogeneic cells from a HLA-matched or HLA-nonmatched live donor or cadaver. The HLA-nonmatched donor may be an unrelated person to the family, but preferably is a very close relative such as one of the parents, a brother or a sister of the patient. In one embodiment, the human cells are T cell-depleted human hematopoietic stem cells prepared by techniques well-known in the art.

The transplanted cells are preferably CD34+ CXCR4+ cells, more preferably CD34+/CD38-/low / CXCR4+ cells.

The hematopoietic human cells for transplantation are preferably obtained from bone marrow or by leukapheresis of peripheral blood from the donor after stimulation by a suitable cytokine such as G-CSF or GM-CSF or each of them in combination with SCF.

The host patient is conditioned under sublethal, lethal or supralethal conditions, for example by total body irradiation (TBI) and/or by treatment with myeloablative and immunosuppressive agents according to standard protocols. For example, a sublethal dose of irradiation is within the range of 3-7 Gy TBI, a lethal dose is within the range of 7-9.5 Gy TBI, and a supralethal dose is within the range of 9-16.5 Gy TBI. Examples of myeloablative agents are busulphan, dimethyl mileran and thiotepa, and of immunosuppressive agents are prednisone, methyl prednisolone, azathioprine, cyclosporine, cyclophosphamide, etc.

The method of the invention is suitable for the treatment of diseases curable by bone marrow transplanttion such as malignant diseases, including leukemias such as acute lymphoblastic leukemia (ALL), acute nonlymphoblastic leukemia (ANLL), acute myelocytic leukemia (AML) and chronic myelocytic leukemia (CML), and severe combined immunodeficiency syndromes (SCID) including adenosine deaminase (ADA) de, osteopetrosis, aplastic anemia, Gaucher's disease, thalassemia and other congenital or genetically-determined hematopoietic abnormalities.

A further application of the invention is in the treatment of other malignant diseases such as breast cancer by purging malignant cells from the patient's blood and transplanting into the patient his own hematopoietic cells purged from the malignant ones. The method is applicable for the types of tumors which malignant cells do not migrate to a chemotactic gradient of SDF-1.

Thus, according to this aspect of the invention, there is provided a method for the preparation of a composition of CXCR4+ cells responsive to SDF-1 for autologous transplantation by ex vivo purging of malignant cells from a cancer patient while maintaining and enriching for normal CXCR4+ stem cells and progenitors, said method comprising:

(i) providing hematopoietic cells from a cancer patient, the malignant cells of which patient do not migrate to a chemotactic gradient of SDF-1;

(ii) stimulating said hematopoietic cells with SCF for up to three days to enhance the CXCR4 expression and response to SDF-1 of said cells;

(iii) carrying out an in vitro transmigration assay with the stimulated cells of (ii) to a gradient of SDF-1 across stromal cells or a stromal cell line;

(iv) washing the migrating cells to remove SDF-1; and

(v) isolating the cells obtained in (iv), said cells being hematopoietic CXCR4+ cells responsive to SDF-1 purged from the patient's malignant cells and suitable for autologous transplantation.

The patient's own hematopoietic cells are derived from the patient's bone marrow or mobilized peripheral blood, e.g. by leukapheresis.

In still another aspect, the invention provides a method for the preparation of a composition of immature hematopoietic cells including stem cells for autologous transplantation for the correction of genetic abnormalities, said method comprising:

(i) introducing a normal gene in CXCR4+ hematopoietic cells responsive to SDF-1 from a patient having a genetic disorder;

(ii) stimulating said stem cells with SCF for up to three days to enhance the CXCR4 expression and response to SDF-1 of said cells;

(iii) carrying out an in vitro transmigration assay with the stimulated cells of (ii) to a gradient of SDF-1 across stromal cells or a stromal cell line;

(iv) washing the migrating cells to remove SDF-1; and

(v) isolating the cells obtained in (iv),

said cells being hematopoietic CXCR4+ cells responsive to SDF-1 containing the normal gene and being suitable for autologous transplantation to correct the patient's genetic disorder.

The hematopoietic cells are derived from the patient's bone marrow or mobilized peripheral blood and are used in a method of autologous transplantation of immature hematopoietic stem cells for gene transfer to correct a patient's genetic disorder, which comprises: (i) conditioning the patient under sublethal, lethal or supralethal conditions; and (ii) transplanting the conditioned patient with the cells.

The invention will now be illustrated by the following non-limiting examples.

DESCRIPTION OF THE FIGURES

Figure 1. Anti-CXCR4 and anti-SDF-1 antibodies inhibit homing of human $CD34^+$ cells to murine bone marrow.

(A) Enriched $CD34^+$ CB cells were pretreated for 30 minutes with anti-CXCR4 antibodies (5 $\mu g/2x10^5$ cells) and injected into NOD/SCID mice ($2x10^5$ cells/mouse). Polyclonal anti-SDF-1 Ab (10 μg/mice) were injected I.V. simultaneously with the cells ($2x10^5$ cells/mouse). Twenty-four hours later the same amount of anti-SDF-1 was injected I.P. As a control the cells were preincubated with anti-CD34 antibodies (5 $\mu g/2x10^5$ cells). The NOD/SCID mice were sacrificed after two weeks and the levels of human progenitors were determined by human-specific semi-solid colony-forming assay (CFU-GM (□), BFU-E (□), and CFU-GEMM (▨)).

(B) Control $CD34^+$ -enriched cord blood cells preincubated with anti-CD34 antibodies were injected into NOD/SCID mice (5 $\mu g/2x10^5$ cells/mouse). Anti-CXCR4 antibodies (12g5, 10 μg/mouse) were injected 30 min, 24 and 96 hr. post-transplantation. After two weeks NOD/SCID mice were sacrificed and the levels of human engraftment were quantified by southern blot using a human-specific probe. A) Average of 3 different experiments ± standard error (SE). B) DNA extracted from the bone marrow of transplanted mice. Each lane represents DNA from one mouse.

(C-E) Prolonged CXCR4 desensitization and down-regulation prevents engraftment of NOD/SCID mice. Enriched $CD34^+$ CB cells were treated for 24 hr. with either SDF-1 (2 μg/ml), PMA (100 ng/ml) or left untreated (CTRL). (C) Treated and untreated cells were then immunostained for CXCR4. (D) Ability of the cells to migrate in response to SDF-1 (125 ng/ml) in a transmigration assay. The percent background migration of untreated control cells with and without SDF-1 (CTRL- and CTRL+, respectively) and the migration of cells pretreated with SDF-1 and PMA to SDF-1 are shown. (E) Percent engraftment of SDF-1-treated cells. Untreated cells or cells treated with SDF-1 were transplanted into NOD/SCID mice ($2x10^5$ cells/mouse) and their engraftment percentage was determined, after 4 weeks, by immunostaining with anti-human CD45 mAb. The results shown in C and D

represent the mean average of 3 different experiments ± SD. The results shown in E are triplicates from one representative experiment out of 3.

Figure 2. SDF-1 induces the migration of SCID repopulating cells.

(A) Migration of $CD34^+$ cells in response to a SDF-1 gradient. Transmigration assay was performed using $CD34^+$ -enriched CB or BM cells. The background migration without SDF-1 (CTRL) and the migration of $CD34^+$ cells in response to a gradient of SDF-1 are shown.

(B) Colony-forming assay of cells migrating in response to SDF-1. The cells migrating in response to SDF-1 (125ng/ml) into the lower chamber (Down), and the cells that remained in the upper chamber (Up) were assayed for colony-forming potential; CFU-GM (□), BFU-E (□), and CFU-GEMM (▨).

(C) Migrating and non migrating $CD34^+$ cells were transplanted into NOD/SCID/b2-M KO mice (3×10^4cells/mouse). After 2 weeks the bone marrow of the transplanted mice was collected, and the levels of engraftment were determined by by immunostaining with anti-human CD45 mAb.

(D) Levels of human progenitors in the murine bone marrow. Levels of human progenitors in the murine bone marrow were determined by human-specific semi-solid colony-forming assay (CFU-GM (□), BFU-E (□), and CFU-GEMM (▨)).

(E) Levels of engraftment in the marrow of NOD/SCID or NOD/SCID b2-M KO mice 2 weeks after transplantation with $3x10^4$ migrating $CD34^+$ CB cells. Each lane contains DNA extracted from the marrow of one mouse. The results shown in A represent the mean average of 11 different experiments ± SD. The results shown in B are from one representative experiment out of 5 and represent the mean average of triplicates ± SD. The results shown in C and D are from one representative experiment out of 3 and represent the mean average ± SD obtained from three mice. In panel E, each lane contains DNA extracted from the bone marrow of one mouse. One typical experiment out of 4 is shown.

Figure 3. SDF-1 preferentially induces the migration of $CD34^+/CD38^{-/low}/CXCR4^+$/SCID repopulating cells.

Surface expression of CD34/CD38 in CB (panel A) or BM (panel B) cells migrating in response to SDF-1. Cells enriched for CD34 migrating in response to SDF-1 (125 ng/ml) into

the lower chamber (Down), and cells that remained in the upper chamber (Up) were collected and immunostained for CD34/CD38 cell surface expression. (C) Engraftment by migrating $CD34^+/CD38^{-/low}$ sorted cells. Transmigration assay was performed using pooled sorted $CD34^+/CD38^{-/low}$ CB cells from two donors. Sorted cells that migrated in response to SDF-1 (125 ng/ml) into the lower chamber and cells that remained in the upper chamber were transplanted into NOD/SCID mice ($3x10^4$cells/mouse). After 6 weeks, bone marrow cells from mice transplanted with migrating sorted cells (I, III and IV) and mice transplanted with nonmigrating sorted cells (II) were pooled and analyzed for levels of human CD34/CD38 cells (C, I, II). R1 gates the $CD34^+/CD38^-$ stem/progenitor cell population. Mice transplanted with migrating sorted cells (C, I, 37%) also contained primitive $CD34^+/CD38^-$ stem/progenitor cells (R1=2.0%), human lymphoid $CD45^+/CD19^+$ pre-B cells (C, III=17%) and non-lymphoid cells (C, III=20%). Marrow from these mice also contained lymphoid progenitors for human $CD56^+$ natural killer cells which were obtained after incubating $2x10^5$ cells with SCF (100 ng/ml) and IL-15 (100 ng/ml) for 10 days (C, IV=87%). The results shown in A represent the mean average of 3 different experiments ± SD. The results shown in B and C are from one representative experiment out of 4.

Figure 4. Stem cell factor potentiates the migration of mobilized $CD34^+$ peripheral blood and cord blood $CD34^+$ cells towards a chemotactic gradient of SDF-1 and enhances their engraftment potential.

(A) Surface expression of CXCR4 in purified $CD34^+$ MPB Cells were either untreated (0 hr.) or treated for 16 or 40 hours with SCF (50 ng/ml) and assayed for surface expression of CXCR4 by immunostaining. (B) Migration percentage of untreated $CD34^+$ MPB (0 hr.) and of cells pretreated with SCF for 16 or 40 hours to a gradient of SDF-1 (125 ng/ml). (C) Percentage of engrafted human MPB $CD34^+$ cells. MPB $CD34^+$ cells were transplanted into NOD/SCID mice ($2x10^5$ cells/mouse) without (0 hr.) or after pretreatment with SCF (50 ng/ml) for 16 or 40 hours. The murine bone marrow was collected after 4 weeks and the percentage of human cells was determined by immunostaining with anti-human CD45 mAb. (D) The same procedure as in C, was repeated in this experiment, however only half of the

number of cells ($1x10^5$cells/mouse) transplanted at time 0 and after treatment with SCF for 16 hours ($2x10^5$cells/mouse) were transplanted after 40 hours of treatment. The levels of human engraftment after one month were quantified by southern blot analysis. (E) Induction of CXCR4 expression in $CD34^+$-enriched CB cells after treatment with SCF (50 ng/ml) for 16 hours (plain line) and 40 hr. (bold line). Cells stained with isotype-matched control mAb are shown (grey bars). (F). Treatment of $CD34^+$ CB cells with SCF enhances their ability to migrate in response to SDF-1 (125 ng/ml) and to engraft NOD/SCID mice. The results shown in A and B represent the mean average of 3 different experiments ± SD. The results shown in C and D are from one representative experiment out of 3 done in duplicates, (D) or triplicates (C). In D each lane contains DNA extracted from the bone marrow of one mouse. The results shown in E and F are from one representative experiment out of 4.

Figure 5. SDF-1 induces shear-resistant adhesion of $CD34^+$/$CXCR4^+$ cells to ICAM-1.

Purified $CD34^+$ CB cells briefly treated with 3μg/ml SDF-1 (◆), 100 ng/ml PMA (O), or left untreated (□) were perfused into a parallel plate flow chamber and allowed to settle for 1 minute at 37^0°C on substrates coated with either ICAM-1-Fc fusion protein immobilized on protein A (A) or sVCAM-1 directly coated on the plate (B). Treatment with SDF-1 together with EDTA (Δ) totally abolished binding of $CD34^+$/$CXCR4^+$ cells to ICAM-1. Following attachment, flow was initiated and increased in 2- to 2.5-fold increments every 5 seconds. The number of bound cells at the end of each shear flow interval was expressed as percentage relative to the number of attached cells prior to flow initiation. Upon initiation of flow, all cells detached immediately from control substrates coated with HSA or protein A (data not shown). The data in (A) and (B) represent the average of three experiments ± SE.

(C-E) Contribution of β1 and β2 integrins to in vitro migration through ECM and engraftment in vivo of $CD34^+$ cells. (C) Percent polarization of purified $CD34^+$ CB cells applied to migration chambers containing ECM-like 3-D gels. (D) Percentage of cells migrating towards a gradient of SDF-1. Untreated cells (Δ) cells treated with a gradient of SDF-1 (◆) and $CD34^+$ cells pre-stained with anti-VLA-4 mAb (5 mg/ml) (O) or anti-VLA-5 mAb (5 mg/ml) (□) and treated with a gradient of SDF-1 are shown in D and E. Purified $CD34^+$ CB cells

were pre-treated with anti-LFA-1, -VLA-4, and -VLA-5 mAbs (5 μg/ml) for 30 minutes and transplanted into NOD/SCID mice. Levels of engraftment were estimated by immunostaining with anti-human CD45 mAb (E). The results shown in C and D represent the mean average of 3 different experiments ± SD. In panel E, each point represents data obtained from one mouse and results were pooled from three different experiments.

Figure 6. A model for homing and engraftment of $CD34^+$ hematopoietic stem/progenitor cells into the bone marrow.

(A) $CD34^+$ cells are recruited to specialized sites on the bone marrow vessel wall, possibly through rolling interactions on constitutively expressed endothelial selectins. (B) Following rolling, $CXCR4^+$ /$CD34^+$ cells (blue cells) are activated by SDF-1 secreted from bone marrow stromal cells. Activation with SDF-1 triggers LFA-1 to support firm adhesion to endothelial ICAM-1. $CD34^+$ cells which do not express sufficient levels of the chemokine receptor CXCR4 (purple) will detach from the endothelial layer and return to the blood stream. (C) The arrested human stem/progenitor cells, in response to a gradient of SDF-1, will extravasate and migrate through the underlying extracellular matrix using their VLA-4 and VLA-5 integrin receptors for fibronectin. (D) Migrating cells will eventually reach "stem cell niches" consisting of stromal cells presenting the proper set of adhesion molecules (e.g. VCAM-1, ICAM-1) and growth stimulatory factors.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

Experimental Procedures

Human cells: Human cord blood (CB) cells were obtained from full term deliveries after informed consent. Mobilized peripheral blood (MPB) and bone marrow (BM) cells were obtained from left over clinical allogeneic harvests from healthy donors, after informed consent. Human cells were used in accordance with approved procedures by the human experimentation and ethics committees of the Weizmann Institute. MPB cells were collected after 5 days of in-vivo treatment with G-CSF and SCF. The blood samples were diluted 1:1 in phosphate-buffered-saline (PBS), supplemented with 1% fetal calf serum (FCS) (Bet Haemek, Israel). Low density mononuclear cells (MNC) were collected after standard separation on Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden), and washed in RPMI with 1% FCS.

Enrichment of human $CD34^+$ cells: Enrichment of human $CD34^+$ cells was performed with a magnetic beads separation kit (mini MACS, Miltney Biotec, Bergisch Glodbach, Germany) according to the manufacturers instructions. The purity of the enriched $CD34^+$ cells was 60-85% when cells were passed over one column, and >98% over two columns. Cells were used directly after enrichment as indicated or frozen in 90% FCS with 10% DMSO.

Flow cytometry analysis: Analysis of human cells in the murine bone marrow. Bone marrow cells from both femurs, tibias, humerus and pelvis bones from each transplanted mouse were flushed with a syringe and 26 gauge needle. Single cell suspensions ($1x10^6$ cells/ml) were washed with PBS supplemented with 1% FCS and 0.02% azide, after red blood cells were lysed with ammonium chloride. For immunostaining, 10^5 cells were resuspended in staining buffer (PBS, 0.1% bovine serum albumin (BSA), 0.02% sodium azide) incubated with 10μg/ml (1:50) of purified anti-mouse CD16/CD32(FcR) (Pharmingen, San Diego, CA, U.S.A) and 1% human plasma for 20 minutes at 4^0C. Cells were then stained with human specific, direct labeled antibodies and incubated for 30 minutes on ice. Isotype control antibodies were used in order to exclude false positive cells and murine bone marrow cells from non transplanted mice were used as negative control cells. Human cells were used as a positive control. Dead cells were gated out by staining with propedium iodide (Sigma, St. Louis, MO, USA). Human cells from engrafted mice were analyzed for immature cells by double staining with anti CD34 FITC (BD) and anti CD38 PE (Coulter) and for the presence of pre B lymphoid cells by staining with anti CD45 FITC (IQP, Holland) and anti CD19 PE (Coulter). The levels of CXCR4 expression on human $CD34^+$ cells were detected with anti CXCR4 PE (Pharmingen) together with anti CD34 FITC (BD). The levels of immature cells in the transwell migration assay were analyzed by staining with anti CD34 FITC (BD) and anti CD38PE(Coulter). The presence of human NK cells in cultures from transplanted mice were detected with anti CD56 PE (Coulter) and anti CD45 FITC (IQP). After staining, cells were washed twice in the same buffer and analyzed on a FACScort (Becton Dickinson,CA), using CellQuest software (BD).

Cell sorting: Cell sorting was performed on a FACStar plus (Becton Dickinson,CA) as previously described (Larochelle et al., 1996). In brief, single cell suspensions of human $CD34^+$ enriched cells (MiniMacs) were labeled with anti human CD34 FITC (BD) and anti

human CD38 PE (Coulter) monoclonal antibodies. The purity of sorted $CD34^+/CD38^{-/low}$ and $CD34^+/CD38^+$ cells was >99%.

Liquid cultures : Human $CD34^+$ enriched cells were seeded in 24 wells plates (Costar, Ma, U.S.A), ($0.2\text{-}1x10^6$ in 0.5 ml), containing either serum free media (IMDM, 2% BSA, 20μg/ml human insulin, 40 μg/ml human LDL, 200 μg/ml transferrin, 10^{-4} M 2ME and 10mM Hepes buffer) or RPMI, 10% FCS, and 1% BSA. In addition various combination of cytokines and chemokines were added as indicated. Serum free cultures yielded similar results compared to cultures that contained RPMI, 10% FCS and 1% BSA. The cultures were incubated at 37^0 C in a humidified atmosphere containing 5% CO_2.

Colony assay: Semisolid progenitor cultures were performed as previously described (Lapidot et al. 1992). In brief, the cells were plated in 0.9% methylcellulose (Sigma, St. Louis, MO, USA), 30% FCS, $5x10^{-5}$ 2ME (Sigma), 50 ng/ml SCF, 5 ng/ml IL3, 5 ng/ml GM-CSF (R&D, Minneapolis, USA), and 2 u/ml Erythropoietin (EPO, Orto Bio Tech, Don Mills, ON, Canada). Bone marrow cells from transplanted mice were cultured under conditions selected for growth of human colonies only, by replacing 15% FCS with 15% human plasma. Plating concentrations were: Enriched $CD34^+$ cells - $3x10^3$ cells/ml, bone marrow cells from transplanted mice - 200 $x10^3$ cells/ml. The cultures were incubated at 37^0 C in a humidified atmosphere containing 5% CO_2 and were scored 14 days later.

Mice: NOD-SCID mice (NOD/LtSz $PrKdc^{scid}/PrKdc^{scid}$)(kindly provided by Dr. John Dick, with the approval of Dr. Leonard. Schultz), HSC Toronto, Ontario, Canada) and NOD/SCID β2-microglobulin-knock out mice (NOD/SCID β2M KO) (kindly provided by Dr. Leonard D. Shultz, Jackson Laboratories, Bar-Harbor Maine, U.S.A) were bred and maintained under defined flora conditions in individually ventilated (HEPA filtered air) sterile micro isolator cages (Techniplast , Italy) at the Weizmann Institute. All the experiments were approved by the animal care committee of the Weizmann institute. Mice, 8 weeks old, were irradiated with a sublethal dose of 375 cGy - 67cG/min. from a cobalt source prior to transplantation. Human $CD34^+$ enriched cells were injected into the tail vain of irradiated mice in 0.5 ml of RPMI with 10% FCS. For in-vivo blocking experiments the cells were first

preincubated with 10μg/ml of anti CXCR4 antibodies (R&D Systems, MBA171 mAb, or Pharmingen, 12g5mAb), or with 10μg/ml of anti VLA-4, VLA-5 and LFA-1 antibodies (Serotec, Oxford, UK), or with anti human CD34 antibodies (Becton Dickenson) as a negative control, for 30 minutes on ice. The cells were then washed and injected into mice. Alternatively transplanted mice were I.P injected with anti CXCR4 or anti SDF-1 antibodies (R&D Systems) as indicated. Mice were sacrificed after 14 - 45 days post transplantation as indicated.

DNA analysis: High-molecular-weight DNA was obtained from the bone marrow and spleens of transplanted mice by phenol/chloroform extraction. DNA (5 μg) was digested with EcoRI, subjected to electrophoresis on 0.6% agarose gel, blotted onto a nylon membrane, and hybridized with a human chromosome 17-specific α-satellite probe (p17H8) labeled with P^{32}. The intensity of the characteristic human 2.7 Kb band in the samples was compared in multiple exposures with that of artificial human mouse DNA mixtures (0%, 0.1%, 1%, and 10% human DNA) in order to quantify the levels of human DNA.

Chemokines and Chemotaxis Assay: Chemotaxis experiments with human $CD34^+$ cells (>98% purity) were assayed by using Costar Transwells (Cambridge, MA, 6.5 mm/diameter, 5 mm/pore). One hundred microliters of chemotaxis buffer (RPMI 1640, 1% FCS) containing $2x10^5$ cells were added in the upper chamber, and 0.6ml of chemotaxis buffer both with or without different concentrations of SDF-1 were added to the bottom chamber. Cells migrating within 4-5 hr. to the bottom chamber of the transwell were counted for 30 seconds using the FACSort (B.D). SDF-1 was purchased from R&D System (Minneapolis, U.S.A).

Controlled Detachment Adhesion Assay: Laminar flow assays were performed as previously described (Carr et al., 1996). In brief, adhesive ligands were diluted in PBS supplemented with 50 mM tris (pH 9.0) and adsorbed on polystyrene plates for 2 hr. at 37^0C, washed three times with PBS, and blocked with 2% HSA in PBS overnight at 4^0C. The plates were assembled as the lower wall of a parallel wall flow chamber and mounted on the stage of an inverted microscope. CB $CD34^+$ cells ($2x10^6$/ml, purity >98%) were suspended in a binding buffer (HBSS, containing 10 mM HEPES (ph 7.4) 1mM Mg^{2+}, 2mM Ca^{2+}, and 2μg/ml HSA), perfused into the chamber and allowed to settle on the substrate coated

chamber wall for 1 min. at 37^0C, and then flow was initiated and increased in 2 to 2.5 fold increments every 5 sec. to generate controlled shear stresses on the wall. Cells were visualized in a 20x objective of an inverted phase-contrast Diaphot Microscope (Nikon, Japan) and photographed with a long integration LIS-700 CCD video camera (Applitech; Holon, Israel), connected to a video recorder (AG-6730 S-VHS, Panasonic, Japan). The number of adherent cells resisting detachment by the elevated shear forces was determined after each interval by analysis of videotaped cell images, and was expressed as the percent of originally settled cells.

To test the effects of SDF-1 or PMA, cells were suspended in binding medium containing 3μg/ml of SDF-1 or 100 ng/ml PMA (Sigma), seconds before being perfused into the chamber. All adhesion experiments were performed at least three times on multiple test fields that contained 50-100 cells/field.

Real-time tracking of $CD34^+$ cell migration in 3D extracellular matrix (ECM)-like gels (Franitza et al., 1998): Purified (>98%) CB $CD34^+$ cells were suspended in a 5 ml drop of a gel-like medium consisted of collagen type I (CO-I), laminin (LN, Cellagen; ICN Pharmaceuticals Inc., CA) and fibronectin (FN, Chemicon; Temecula), in RPMI (at final concentrations of 1.8 mg/ml; 6 and 2.5 mg/ml, respectively). A second drop without cells was placed 1.5 mm from drop I. An SDF-1 depot was created in a third gel-like drop supplemented with SDF-1 (500ng/ml), placed 1.5 mm downstream of drop II and 3-5 mm from drop I. Once the three gelatinous drops started to polymerize, the drops were gently connected with a fine needle to form a continuous 3D gel and cell migration within this gel was tracked by time-lapse videomicroscopy. Cell images were visualized as described above and videotaped on a time-lapse video recorder (AG-6730 S-VHS, Panasonic) at 25 frames per min.

Cell locomotion was analyzed manually from played-back video segments. $CD34^+$ cell positions in a representative field of view were tracked for 60-90 min. Time zero (t=0) was set according to the time at which the cells located at the edge of the field closest to the hemoattractant source started to spread and polarize, in response to the diffused chemoattractant. In representative experiments, FITC-dextran (10 kDa; Sigma) was introduced into drop I and used as a marker to monitor the rate of chemokine diffusion within the connected drops. Cellular movements were assigned as follows: stationary cells with polarized morphology (polarized), motile cells that moved randomly in the gel or in a direction away from the chemoattractant (randomly migrating cells), and cells that migrated towards the

source of the chemoattractant (directionally moving cells). The proportions of polarized, non-motile, randomly migrating, and directionally migrating cells within the entire population of cells in the field were determined for six intervals within the time of 60-90 min. of tracking. The role of specific β1 integrins in human $CD34^+$ cell migration was examined by preincubating (10 min, 4^0C) $CD34^+$ cells (10^6/ml) in a 200 ml RPMI mixture containing 1% BSA and 5 μg of a control isotype-matching mAb, and then incubating (20 min, 4^0C) with specific murine mAbs to the α4 (CD49d; IgG1), α5 (CD49e; IgG2A) of the β1 integrins (Serotec, Oxford, UK). Subsequently the $CD34^+$ cells were extensively washed and added to the 3-D gels. When injected into mice, $CD34^+$ cells (60-85% purity) ($2x10^5$/mouse) were preincubated (20 min., 4^0C) with murine mAb to the human adhesion antigens LFA-1(CD11a) α4 (CD49d; IgG1), α5 (CD49e; IgG2A), of the β1 and β2-integrins (Serotec).

Example 1

Anti SDF-1 and anti CXCR4 antibodies inhibit homing of human $CD34^+$ cells into the murine bone marrow

To examine the possible in-vivo role of SDF-1 and its receptor CXCR4 in migration and engraftment of human SRC we pretreated $CD34^+$ enriched cord blood cells with two different anti CXCR4 antibodies or with anti CD34 antibodies as a control. Both anti CXCR4 antibodies, but not anti CD34 antibodies, dramatically decreased the engraftment levels (Fig. 1A). Similar pretreatment of human $CD34^+$ enriched cells from adult bone marrow or mobilized peripheral blood (PB) also resulted in an inhibition of engraftment (data not shown). Anti SDF-1 antibodies co-injected with human $CD34^+$ cord blood cells and re-administered after 24 hours, also significantly decreased the level of engraftment (Fig. 1A). Kinetic experiments in which anti CXCR4 antibodies were administered at varying points in time after transplantation revealed that the first 24 hours time period is critical in the engraftment process. When the antibodies were administered by I.V injection together with the cells (Fig. 1A) or 30 minutes after transplantation by I.P injection, they almost totally blocked engraftemnt (Fig. 1B). Antibodies administered by I.P. injection 24 hours later, reduced engraftment, although less effectively (Fig. 1B) and were completely ineffective when

administered 4 days following transplantation (Fig. 1B). Treatment of $CD34^+$ cells with anti CXCR4 antibodies did not cytotoxically damage the cells, as judged by their normal proliferation in response to cytokine stimulation, and formation of colonies in semi-solid medium (data not shown).

SDF-1 and phorbol esters were reported to cause internalization and down regulation of CXCR4 surface expression on human $CD4^+$ T cells (Signoret et al., 1997; Haribabu et al., 1997; Amara et al., 1997). These agents also reduced cell surface expression of CXCR4 on human $CD34^+$ cells within 30 minutes of addition (data not shown). To study the effects of SDF-1 desensitization and CXCR4 down regulation on the ability of human $CD34^+$ cells to migrate and engraft NOD/SCID mice, $CD34^+$ enriched cord blood cells were incubated over night with high doses of SDF-1 (2μg/ml). The cells were subsequently washed and assayed for CXCR-4 expression and migration. Pretreatment with PMA or SDF-1, reduced CXCR4 cell surface expression (Fig. 1C), and abolished the migration of $CD34^+$ cells in response to SDF-1 (Fig 1D), without affecting the ability of the cells to form colonies in vitro (data not shown). In three independent experiments, prolonged (24 hr.) treatment of $CD34^+$ cells with high doses of SDF-1 significantly blocked the engraftment of transplanted NOD/SCID mice (Fig. 1E).

Example 2

SDF-1 preferentially induces migration and engraftment of $CD34^+/CD38^{-/low}/ CXCR4^+$ cells

It is concluded therefore that the antibodies to CXCR4 or SDF-1 desensitization and CXCR4 internalization, interfered with one or several steps in the engraftment process. Further experiments were therefore aimed at analyzing the target cells for these antibodies and the mechanism by which the chemokine and its receptor mediate hemopoietic repopulation.

The migration potential of human $CD34^+$ cells was tested in-vitro in a transwell migration assay. Human $CD34^+$ enriched cells from cord blood, bone marrow and mobilized PB were added to the upper chamber and SDF-1 to the bottom chamber (125ng/ml). Consistent with results of previous studies (Aiuti et al., 1997; Kim and Broxmeyer 1998), we

also found that 20-25% of cord blood and bone marrow $CD34^+$ cells migrated in response to a gradient of SDF-1 (Fig. 2A). The ability of mobilized PB $CD34^+$ cells taken from 9 donors to migrate in response to SDF-1 was highly variable ranging between 8% to 60%. No differences were observed in the incidence of progenitor cells, as judged by in vitro colony assays, comparing the migrating and non migrating cord blood $CD34^+$ cells (Fig. 2B), as well as bone marrow and mobilized PB $CD34^+$ cells (data not shown). The engraftment potential of the migrating and non migrating $CD34^+$ cells was further examined. Equal numbers of $CD34^+$ enriched cord blood cells from the upper and lower chambers were washed and transplanted into NOD/SCID or NOD/SCID β2 M KO mice. While mice that were transplanted with cells form the upper chamber had poor levels of engraftment, mice transplanted with cells that migrated to SDF-1 had much higher levels of human cells (Fig. 2C) including multilineage, progenitors (Fig. 2D) and $CD19^+$ Pre B cells (data not shown). Significantly higher engraftment levels were evident in NOD/SCID β2 M KO mice recipients, which have relatively less residual immunity than in NOD/SCID mice recipients (Fig. 2E). Thus, the increased engraftment of $CD34^+$ cells in response to SDF-1 is associated with augmented migration potential.

Cord blood $CD34^+$ cells that migrated to the SDF-1 gradient had a significantly higher percentage of primitive $CD34^+$ /$CD38^-$ cells than cells left in the upper chamber (Fig. 3A), even though the majority of $CD34^+$ /$CD38^-$ cells did not migrate. When human bone marrow $CD34^+$ enriched cells were assayed, the proportion of immature $CD34^+$ /$CD38^{-/low}$ cells migrating to the chemotactic SDF-1 gradient increased (Fig. 3B). Sorted $CD34^+$/$CD38^{-/low}$ cord blood cells from different donors were therefore evaluated for their ability to migrate towards a chemotactic gradient of SDF-1 in vitro and for their content of SRC in vivo. Only 26% (±7%) of the cells from eight different donors migrated to a gradient of SDF-1 in the transwell migration assay. In a typical experiment, transplantation of migrating $CD34^+$/$CD38^{-/low}$ cells into NOD/SCID mice resulted in very high levels of engraftment (Fig. 3CI, 35%). In contrast, only low levels of engraftment were observed with non migrating

$CD34^+$ /$CD38^{-/low}$ cells (Fig. 3CII, 1.2%). High level multi lineage human hematopoiesis was detected in the murine bone marrow repopulated with migrating $CD34^+/CD38^{-/low}/CXCR4^+$ cells. This was reflected in the engraftment of primitive $CD34^+/CD38^-$ cells (Fig. 3CI, r1=2%), $CD45^+/CD19^+$ pre B cells (Fig. 3CIII, 17%) as well as in myeloid, erythroid (data not shown) and NK progenitors (Fig. 3CIV). These results indicate that only $CD34^+/CD38/^{-/low}/CXCR4^+$ cells representing less than one third of all $CD34^+/CD38^{-/low}$ cells, migrate in response to a chemotactic gradient of SDF-1, and engraft the murine bone marrow with SRC.

Example 3

Stem cell factor potentiates the in vitro migration of mobilized peripheral blood and cord blood $CD34^+$ cells towards a chemotactic gradient of SDF-1 and enhances their engraftment potential

Prolonged 24-hour treatment of $CD34^+$ cells with high doses of SDF-1 significantly blocked the engraftment of transplanted NOD/SCID mice (Fig. 1E). Following SDF-1 removal and stimulation with SCF, CXCR4 expression on the surface of human cord blood $CD34^+$ cells was increased, concomitant to recovery of the potential for migration and engraftment (data not shown). Furthermore, stimulation of mobilized human PB $CD34^+$ cells with SCF (50 ng/ml) for 36-48 hours increased CXCR4 expression significantly (Fig. 4A). Concurrent with the increase in CXCR4 cell surface expression, we found a dramatic improvement in the migration of the cells across a gradient of SDF-1 in transwell migration assays (Fig. 4B). In parallel, a time dependent improvement in the engraftment potential of mobilized PB $CD34^+$ cells was observed (Fig. 4C). A similar increase in engraftment potential was recorded even when only half the cell number was injected after 40 hours of SCF treatment, compared to the potential in twice that many cells that were transplanted at time 0 and after 16 hrs. stimulation with SCF (Fig. 4D). Our data suggests that by increasing CXCR4 expression we have also expanded the population of human SCID repopulating cells. When cord blood $CD34^+$ cells were stimulated with SCF for 36-48 hours, a similar increase in CXCR4 expression (Fig. 4E), migration in response to SDF-1 (Fig. 4F), and higher levels of engraftment (Fig. 4F) were observed. These results demonstrate for the first time that in-vivo engraftment potential correlates with in vitro migration towards a gradient of SDF-1.

More importantly these processes can be enhanced by stimulation with SCF, resulting in increased cell surface CXCR4 expression, migration and engraftment. Taken together our data establish a functional animal model which can be used to detect and quantify the migration and engraftment potential of immature human cells as well as to identify chemokines and cytokines which regulate these processes.

Example 4

SDF-1 induces firm LFA-1 mediated adhesion of $CD34^+$ /$CXCR4^+$ cells to ICAM-1, but fails to activate VLA-4 or VLA-5 adhesion to VCAM-1 and fibronectin.

In order to further understand the mechanism by which SDF-1 regulates migration and engraftment, we tested the direct effect of SDF-1 on the ability of both β1 and β2 integrins to develop firm adhesion of cord blood $CD34^+$ cells to VCAM-1, ICAM-1 and fibronectin (FN). Integrin dependent adhesion assays were performed using a parallel-plate flow chamber, which simulates blood flow and allows the application of both weak and strong detaching forces on adherent cells (Carr et al., 1996). Highly purified $CD34^+$ cells (>98%), treated briefly with SDF-1 and with PMA or left untreated, were allowed to bind for 1 minute to immobilized VCAM-1 or ICAM-1 in stasis. The cells were then subjected to incremented shear flow which generated increasing detaching forces on the adherent cells. SDF-1 rapidly activated the firm shear-resistant adhesion of $CD34^+$/$CXCR4^+$ cells to immobilized ICAM-1 (Fig. 5A). Chemokine-mediated activation was almost as powerful as activation with the nonphysiological integrin agonist PMA, and was integrin dependent as it was totally inhibited by the addition of EDTA (Fig. 5A). Surprisingly, unlike with human $CD4^+$ T cells, SDF-1 failed to stimulate the VLA-4 dependent $CD34^+$ cells adhesion to VCAM-1 (Fig. 5B), which suggests that SDF-1 can activate LFA-1 without stimulating VLA-4 adhesion on the same cell population. All cellular interactions were videotaped and individually analyzed. In parallel experiments, we used moderate shear forces to assess the adhesive strength of $CD34^+$ cells that statically adhered to fibronectin or to certain integrin-binding fibronectin fragments FN40 (contains the VLA-4 binding site), and FN120 (contains the VLA-5 binding site). We found that brief exposure to SDF-1 failed to stimulate or even marginally elevate VLA-4 or VLA-5 mediated adhesion (data not shown). The binding potential of $CD34^+$ cells to VCAM-1, to

fibronection and to the various integrin binding fibronectin fragments was dramatically induced by PMA as well as by the stimulatory anti integrin mAb TS 2-16. The latter is a direct activator of integrin affinity to its ligand, and augments the potential of different integrins sharing the same β1 subunit to adhere (Fig. 5B, and data not shown). These results indicate that the integrins VLA-4 and VLA-5 can be functionally activated on $CD34^+$ cells by inside out signaling, distinct of CXCR4.

Adhesion molecules are involved in the interactions between $CD34^+$ cells and bone marrow extracellular matrix (ECM) as well as between $CD34^+$ cells and stromal cells (Teixido and Anklesaria, 1992; Quesenberry et al., 1991). We studied the migratory properties of CB $CD34^+$ cells through a 3-dimensional (3-D) ECM-like gel, reconstituted with a meshwork of collagen, FN and laminin (Franitza et al., 1998), to which an SDF-1 gradient was introduced. This novel system allows for the close examination of the random and directional migration of cells towards a newly generated chemoattractant source in real time. Most $CD34^+$ cells embedded in this gel remained spherical and failed to polarize or migrate in the absence of SDF-1 (Fig. 5C). However, upon introduction of an SDF-1 gradient, 45%-50% of the cells polarized in a time dependent manner (Fig. 5C). As much as 70% of these cells migrated towards a gradient of SDF-1 (Fig. 5D). Polarization and movement correlated with both the level of CXCR4 expression on $CD34^+$ cells, and with their transmigration capacity along a gradient of soluble SDF-1 (data not shown). Although SDF-1 did not mediate VLA-4 and VLA-5 adhesion, SDF-1-induced polarization and directional movement in ECM-like gels seem to be greatly dependent on VLA-4 as well as VLA-5 integrins, as observed in the inhibition of these processes by neutralizing antibodies to each of these integrins (Fig. 5C, D).

To further determine the in-vivo roles of LFA-1, VLA-4, and VLA-5, in migration and engraftment of human SRC, $CD34^+$ enriched cord blood cells were pretreated with antibodies against one of the above integrins or as a control with anti CD34 antibodies. As expected, anti LFA-1, anti VLA-4, and anti VLA-5, antibodies all blocked the engraftment of $CD34^+$ cells to the mouse bone marrow, while control anti CD34 antibodies did not (Fig. 5E). Our *in vitro* and in-vivo results suggest a crucial role for integrins in the multi step process of migration and engraftment by human SRC.

DISCUSSION

Functional in-vivo assays for primitive human SCID repopulating cells, that provide a means to measure the engraftment properties of various types of human hematopoietic cells, have been developed in recent years by us and by others (Lapidot et al., 1992; Larochelle et al., 1996; Cashman et al., 1997; Dick et al., 1997) One major area of interest concerns the elucidation and characterization of processes that regulate migration of human hematopoietic stem/progenitor cells and their homing to the bone marrow. Knowledge of this sort could then be applied in further research to develop a means of enhancing the incidence of stem cells with migration and engraftment potential.

In the present invention we identified the chemokine SDF-1 as a key mediator of migration and engraftment of human $CD34^+/CXCR4^+$ cells to the bone marrow of immune deficient mice. This is the first assignment of a distinct in-vivo function of SDF-1 using human cells. We further identified a functional internal hierarchy within the early population of $CD34^+/CD38^{-/low}$ cells. In this population a minority of the primitive cells that were $CXCR4^+$ and that migrated to a gradient of SDF-1 in vitro, repopulated the murine bone marrow with SRC. Our data characterizes SRC as $CD34^+/CD38^{-/low}/CXCR4^+$ cells with major stem cell properties. We thus redefine human SRC/stem cells as those with a $CXCR4^+/CD38^{-/low}$ phenotype and with the potential to migrate to a gradient of SDF-1 and to engraft the murine bone marrow with high levels of myeloid and lymphoid cells.

Based on these findings we suggest that in-vivo there is a steady-state balance between a minority of $CD34^+$ cells that express sufficient levels of CXCR4 and consequently migrate to SDF-1, with a majority of the cells that express low levels of CXCR4 or do not express the receptor at all and therefore can not migrate. In support of our findings a recent study demonstrated that over expression of human CXCR4 receptor in murine T cells led to enhanced migration of these cells to the murine bone marrow (Koito, 1998). This balance may be controlled by cytokines such as SCF which upregulates CXCR4 expression as well as by SDF-1 which inhibits CXCR4 expression after migration. Interestingly this balance is also maintained in the primitive population of $CD34^+/CD38^-$ and $CD34^+/CD38^{-/low}$ cells, which contain a higher fraction of $CXCR4^+$ migrating cells than the more differentiated

$CD34^+/CD38^+$ cells that were isolated from bone marrow or cord blood. The above balance may reflect the production of SDF-1 in the bone marrow which in turn controls the localization of stem cells and their progeny within the marrow microenvironment. A similar mechanism has been suggested for T cells in lymph nodes (Bleul et al., 1998). Mobilization of $CD34^+$ cells from the bone marrow to the blood circulation by cytokine stimulation alters this balance, resulting in heterogeneous levels of CXCR4 expression.

SCF, an early acting cytokine with many hematopoeitc functions, has a membrane bound form expressed on stromal cells as well as a soluble form (Zsebo et al., 1990; Kapur et al., 1998). We demonstrate that prolonged stimulation of mobilized PB, cord blood and bone marrow $CD34^+$ cells, with SCF, significantly increased CXCR4 cell surface expression, as well as migration and bone marrow engraftment. In a recent study, the prolonged stimulation of human $CD4^+$ T cells with IL-4 was shown to increase cell surface CXCR4 expression (Jourdan et al., 1998). These findings suggest that the population of migrating human SRC/stem cells can be expanded ex-vivo by upregulating CXCR4 expression. Stimulation with SCF could be applied to improve human stem cell transplantation protocols. Furthermore, it might be possible to purge *in-vitro* malignant cells in cases of disease that require autologous transplantation, provided that the malignant cells do not migrate to a gradient of SDF-1. Kim and Broxmeyer have demonstrated that SCF can increase the motility of $CD34^+$ cells and also has low chemotactic and chemokinetic activities on human $CD34^+$ cells (Kim and Broxmeyer, 1998). Another study demonstrated that applying prolonged in-vitro exposure to a gradient of SCF results in migration of murine stem cells (Okumura et al., 1996). SCF was moreover found to synergize with SDF-1, resulting in significantly increased levels of migration by human $CD34^+$ cells to a mixture of these chemoattractants in-vitro (Kim and Broxmeyer, 1998). These effects may also be due to increases in surface CXCR4 expression, since mice that lack SDF-1 or CXCR4 expression lack hematopoiesis in the fetal bone marrow while mutant Sl/Sl mice, that lack SCF, or W/W^v mice, which have a defective ckit receptor, have both myeloid and lymphoid marrow hematopoiesis (Nagasawa et al., 1996; Nagasawa, 1998; Zou et al., 1998; Zsebo et al., 1990).

Adhesion molecules are involved in the interactions between $CD34^+$ cells and the extracellular matrix as well as between $CD34^+$ cells and stromal cells (Teixido and Anklesaria, 1992; Quesenberry et al., 1991). These interactions are pivotal in the process of homing and engraftment. The involvement of SDF-1 in the rapid physiological shift from rolling behavior on endothelial cells lining the blood vessels, to firm ICAM-1/LFA-1 dependent arrest of human $CD4^+$ T lymphocytes suggests a similar mechanism of action for SDF-1 in the control of migrating $CD34^+/CXCR^+$ cells (Campbell et al., 1998; Laudanna et al., 1996). Indeed neutralizing antibodies to LFA-1 could prevent engraftment by human $CD34^+$ cells. Preliminary results indicate that human $CD34^+$ cells can also roll on P selectin, possibly as a preceding step before interacting with ICAM-1 (data not shown). We have also found that SDF-1 is capable of activating shear-resistant adhesion of $CD34^+/CXCR^+$ cells to ICAM-1. However, in contrast to ICAM-1, we did not find any effect of SDF-1 on binding of $CD34^+$ cells to VCAM-1, or fibronectin. Using a novel 3-dimensional (3-D) ECM-like gel, we found that directional migration of $CD34^+/CXCR4^+$ cells towards a chemotactic gradient of SDF-1 was dependent on VLA-4 as well as on VLA-5. Furthermore, we showed in-vivo that neutralizing antibodies to VLA-4, and VLA-5 block the migration and engraftment of SRC/stem cells in murine bone marrow. These findings suggest that VLA-4 and VLA-5 interactions between human $CD34^+/CXCR4^+$ cells and the extracellular matrix are critical for migration of stem cells to the bone marrow and successful engraftment. Post migration adhesion to stromal cells by activation of VLA-4 and VLA-5, could be mediated by other cytokines such as SCF which was found to induce in vitro adhesion of human $CD34^+$ cells to fibronectin via transient activation of VLA-4 and VLA-5 (Levesque et al., 1995).

Based on our data we suggest the following possible scenario for homing of human SRC/stem cells to the bone marrow: Within the first 24 hr. after transplantation, $CD34^+/CD38^{-/low}/CXCR4^+$ SRC/stem cells that express P selectin ligand (Spertini et al., 1996), reach the bone marrow and are recruited to specific vascular sites which constitutively express P selectin. Upon activation with endothelium expressed SDF-1, LFA-1 is activated on rolling stem cells to support their firm adhesion to the vessel wall. In response to a gradient of

SDF-1 the arrested human stem cells polarize and extravasate the bone marrow hemopoietic compartment (diapedesis). By using VLA-4 and VLA-5, the cells move towards local gradients of SDF-1, thereby produced by specialized stromal cells, orienting themselves through the different elements of the bone marrow microenvironment and into the "stem cell niches" (Fig. 6)

Our studies suggest that migration, and therefore the engraftment potential of stem/progenitor cells from cord blood, bone marrow, and mobilized PB $CD34^+$ cells is significantly underestimated at the time of harvest. It appears that both in vitro migration as well as homing and engraftment of human $CD34^+/CD38^{-/low}/CXCR4^+$SRC/stem cells can be regulated and increased. While most previous studies have demonstrated the importance of the proliferation and differentiation potential of stem cells as the major criteria for their developmental and repopulation status, our work suggests that CXCR4 dependent migration to SDF-1 is crucial for bone marrow engraftment and repopulation. Therefore we propose that the migration capacity, (which is measured by cell surface CXCR4 expression, level of migration to a chemotactic gradient of SDF-1 and level of engraftment by primitive human $CD34^+/CD38^{-/low}$ cells), as a highly variable factor, plays an equally important role. Results of studies by others which linked expansion or differentiation of repopulating stem cells to engraftment could actually be explained by changes in the migration status of these cells, which reflect alterations in the levels of CXCR4 expression. Of particular importance is the issue of stem cell self renewal. The latter process can be quantified by serial stem cell transplantation's. Previous studies demonstrated a dramatic decrease in the frequency of repopulating mouse stem cells after each passage in-vivo ((Harrison et al., 1990; Spangrude et al., 1995). Among recipients of clinical stem cell transplantation, decreases in long term culture initiating cells were reported (Sutherland et al., 1995). Our results suggest that at least part of the decline in repopulating stem cells is not necessarily due to accelerated differentiation, as previously thought, but rather to loss of the majority of stem cells with migration and engraftment potential which fail to engraft due to low levels or complete lack of cell surface CXCR4 expression.

Our results establish a functional animal model for the in-vivo examination of human hemopoietic cell engraftment in mice, which serves several purposes: 1) Identification of chemokines and cytokines such as SDF-1 and SCF that mediate or regulate migration and

bone marrow engraftment by immature human $CD34^+$ cells. 2) Quantitative measurements of the migration and bone marrow engraftment potential of human $CD34^+/CD38^{-/low}/CXCR4^+$ SRC with major stem cell properties. 3) Characterization of key adhesion molecules such as LFA-1, VLA-4 and VLA-5 and identification of their specific roles in migration and engraftment. 4) Development of ex-vivo protocols for expansion of SRC/stem cells by treatment with specific cytokines that up regulate CXCR4 expression and increase their migration and engraftment potential.

Our findings delineate key steps in the complex engraftment process and suggest upregulation of CXCR4 as a novel approach to expand migrating $CXCR4^+$ stem cells for clinical transplantation. In addition our data may be relevant in designing strategies for prevention of CXCR4 mediated HIV infections.

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CLAIMS

1. A cell composition consisting essentially of a cellular population of viable mammalian immature hematopoietic cells including stem cells, which cells have the following characteristics:

(i) the cells are CXCR4+, namely the cells express or have the potential to express CXCR4, the cell surface receptor of the chemokine stromal-derived factor 1 (SDF-1);

(ii) the cells migrate in response to SDF-1;

(iii) the migration to SDF-1 through extracellular matrix is dependent on VLA-4 and VLA-5;

(iv) in response to SDF-1 the cells can activate LFA-1; and

(v) the cells can be successfully engrafted in a mammal, in which mammal they are capable of proliferation and differentiation into myeloid and/or lymphoid cells;

2. The cell composition according to claim 1, wherein the cells with the potential to express CXCR4, upon stimulation with an agent, express CXCR4 in a time-dependent manner.

3. The cell composition according to claim 2, wherein said agent is a cytokine involved in maintenance, expansion and/or development of stem cells.

4. The cell composition according to claim 3, wherein said cytokine is stem cell factor (SCF).

5. A human cell composition according to any one of claims 1 to 4.

6. The human cell composition according to claim 5, wherein the immature hematopoietic cells are derived from bone marrow, cord blood, fetal liver, yolk sac or mobilized peripheral blood.

7. The human cell composition according to claim 5 or 6, consisting of immature human hematopoietic CD34- CXCR4+ cells.

8. The human cell composition according to claim 5 or 6, consisting of human hematopoietic CD34+ CXCR4+ cells.

9. The human cell composition according to claim 8, consisting of human hematopoietic CD34+/CD38-/low / CXCR4+ cells.

10. A method for preparing a cell composition according to claim 1, comprising:

(i) removing red blood cells and granulocytes from bone marrow, cord blood, fetal liver, yolk sac or mobilized peripheral blood cells;

(ii) treating the cells from (i) to remove mature cells;

(iii) treating the thus obtained enriched population of immature cells from (ii) being CXCR4+ cells and cells with the potential to express CXCR4 with an agent for a determined period of time to enhance CXCR4 expression,

thus obtaining a cell composition consisting of an enriched population of mammalian immature hematopoietic CXCR4+ cells that include stem cells.

11. The method according to claim 10, wherein said agent in step (iii) is a cytokine involved in maintenance, expansion and/or development of stem cells.

12. The method according to claim 11, wherein said cytokine is SCF and the cells are treated with SCF for up to three days.

13. A method for preparing a cell composition consisting of human hematopoietic CD34+ CXCR4+ cells according to claim 8, said method comprising:

(i) removing red blood cells and granulocytes from bone marrow, cord blood, fetal liver, yolk sac or mobilized peripheral blood cells;

(ii) treating the cells from (i) with anti-CD34 monoclonal antibodies to enrich for population of CD34+ cells; and

(iii) treating the thus obtained enriched population of CD34+ cells from (ii) with an agent for a determined period of time to enhance CXCR4 expression,

thus obtaining a cell composition consisting of an enriched population of CD34+CXCR+ cells.

14. The method according to claim 13, wherein said agent in step (iii) is a cytokine involved in maintenance, expansion and/or development of stem cells.

15. The method according to claim 14, wherein said cytokine is SCF and the enriched population of CD34+ cells is treated with SCF for up to three days.

16. The method according to any one of claims 13 to 15, wherein the enriched human hematopoietic CD34+CXCR4+ cell population is further enriched to obtain human hematopoietic CD34+/CD38-/low CXCR4+ cells.

17. The method according to claim 16, wherein said further enrichment to human hematopoietic CD34+/CD38-/low CXCR4+ cells is carried out with anti-CD38 monoclonal antibodies.

18. A chimeric mammal transplanted with a cell composition of claim 1, said chimeric mammal being capable of supporting the proliferation and differentiation of the transplanted immature hematopoietic cells including stem cells into myeloid and/or lymphoid cells.

19. The chimeric mammal according to claim 18, being transplanted with a cell composition consisting of human hematopoietic CD34+CXCR4+ cells.

20. The chimeric mammal according to claim 19, being transplanted with a cell composition consisting of human hematopoietic CD34+/CD38-/low / CXCR4+ cells.

21. The chimeric mammal according to claim 18, wherein the engraftment of the cells is carried out by a process comprising:

(i) sublethally irradiating an immunodefficient mammal lacking a population of functional B and T cells; and

(ii) transplanting into the irradiated immunodefficient mammal the hematopoietic cells of claim 1, optionally after treatment with an agent for a determined period of time to enhance cell CXCR4 expression.

22. The chimeric mammal according to claim 19 or 20, wherein the engraftment of the cells is carried out by a process comprising:

(i) sublethally irradiating an immunodefficient mammal lacking a population of functional B and T cells; and

(ii) transplanting into the irradiated immunodefficient mammal human hematopoietic CD34+CXCR4+ or CD34+/CD38-/low / CXCR4+ cells, optionally after treatment with an agent for a determined period of time to enhance cell CXCR4 expression.

23. The chimeric mammal according to claim 21 or 22, wherein said agent in step (ii) is a cytokine involved in maintenance, expansion and/or development of stem cells.

24. The chimeric mammal according to claim 23, wherein said cytokine is SCF and the human hematopoietic CD34+CXCR4+ or CD34+/CD38-/low/CXCR4+ cells are transplanted into said irradiated immunodefficient mammal 12 to 120 hours after stimulation with SCF.

25. The chimeric mammal according to any one of claims 18 to 24, which is a NOD/SCID or a NOD/SCID β2-microglobulin-knock out (NOD/SCID β2M KO) mouse.

26. Use of a chimeric mammal according to any one of claims 18 to 25 as a model for testing the engraftment efficiency of human immature hematopoietic CXCR4+ cells derived from bone marrow, cord blood, fetal liver, yolk sac or mobilized peripheral blood, which comprises testing the level of human myeloid and lymphoid cells in the mammal after engraftment of the human immature hematopoietic CXCR4+ cells tested.

27. An in vitro method for screening hematopoietic cells as candidates for transplantation into human hosts by testing the migration efficiency of hematopoietic CXCR4+ cells derived

from bone marrow, cord blood, fetal liver, yolk sac or mobilized peripheral blood as cand, said method comprising:

(i) measuring the level of cell surface CXCR4 expression in said cells with labeled anti-CXCR4 monoclonal antibodies; and

(ii) measuring the cells' ability to migrate in response to SDF-1 in a cell migration assay with a gradient of SDF-1, optionally across stromal cells or a stromal cell line,

the cells with a high migratory capability in response to SDF-1 being suitable for successful transplantation into human hosts.

28. A method for testing and ascertaining the engraftment capability of human hematopoietic CXCR4+ cells derived from bone marrow, cord blood, fetal liver, yolk sac or mobilized peripheral blood, which cells have a high expression level of CXCR4 and high migratory capability in response to SDF-1, said method comprising transplanting said cells into a chimeric mammal according to any one of claims 18-25, whereby stable engraftment in the model chimeric mammal capable of supporting the proliferation and differentiation of said transplanted cells into myeloid and/or lymphoid cells, indicate the suitability of said cells for successful engraftment in human hosts.

29. A method for transplantation of immature hematopoietic stem cells in a patient in need therefor, said method comprising:

(i) conditioning the patient under sublethal, lethal or supralethal conditions; and

(ii) transplanting the conditioned patient with a cell composition of claim 1.

30. The method according to claim 29, wherein said comcomprises human immature hematopoietic cells.

31. The method according to claim 30, wherein said human immature hematopoietic cells are derived from bone marrow, cord blood, fetal liver, yolk sac or mobilized peripheral blood.

32. The method according to claim 30 or 31, wherein said human cells are autologous cells or from a HLA-matched or HLA-nonmatched live donor or cadaver.

33. The method according to any one of claims 30 to 32, wherein said human cells are T cell-depleted human hematopoietic stem cells.

34. The method according to any one of claims 30 to 33, wherein said human cells are CD34+ CXCR4+ cells.

35. The method according to any one of claims 30 to33, wherein said human cells are CD34+/CD38-/low / CXCR4+ cells.

36. The method according to any one of claims 30 to 35, wherein said human cells are obtained from bone marrow or by leukapheresis of peripheral blood from the donor after stimulation by a suitable cytokine.

37. The method according to any one of claims 30 to 36, wherein the host patient is conditioned under sublethal conditions.

38. The method according to any one of claims 30 to 36, wherein the host patient is conditioned under lethal or supralethal conditions

39. The method according to claim 38, wherein said lethal or supralethal conditions include total body irradiation (TBI).

40. The method according to claim 38, wherein said lethal or supralethal conditions include TBI followed by treatment with myeloablative and immunosuppressive agents.

41. The method according to claim 32, wherein said lethal or supralethal conditions include treatment with myeloablative and immunosuppressive agents without TBI.

42. The method according to any one of claims 29-41, for the treatment of malignant diseases.

43. The method according to any one of claims 29-42, wherein the transplanted cells are autologous cells.

44. A method for the preparation of a composition of CXCR4+ cells responsive to SDF-1 for autologous transplantation by ex vivo purging of malignant cells from a cancer patient while maintaining and enriching for normal CXCR4+ stem cells and progenitors, said method comprising:

(i) providing hematopoietic cells from a cancer patient, the malignant cells of which patient do not migrate to a chemotactic gradient of SDF-1;

(ii) stimulating said hematopoietic cells with SCF for up to three days to enhance the CXCR4 expression and response to SDF-1 of said cells;

(iii) carrying out an in vitro transmigration assay with the stimulated cells of (ii) to a gradient of SDF-1 across stromal cells or a stromal cell line;

(iv) washing the migrating cells to remove SDF-1; and

(v) isolating the cells obtained in (iv),

said cells being hematopoietic CXCR4+ cells responsive to SDF-1 purged from the patient's malignant cells and suitable for autologous transplantation.

45. A method according to claim 44, wherein the hematopoietic cells are derived from the patient's bone marrow or mobilized peripheral blood.

46. A method for the preparation of a composition of immature hematopoietic cells including stem cells for autologous transplantation for the correction of genetic abnormalities, said method comprising:

(i) introducing a normal gene in CXCR4+ hematopoietic cells responsive to SDF-1 from a patient having a genetic disorder;

(ii) stimulating said stem cells with SCF for up to three days to enhance the CXCR4 expression and response to SDF-1 of said cells;

(iii) carrying out an in vitro transmigration assay with the stimulated cells of (ii) to a gradient of SDF-1 across stromal cells or a stromal cell line;

(iv) washing the migrating cells to remove SDF-1; and

(v) isolating the cells obtained in (iv),

said cells being hematopoietic CXCR4+ cells responsive to SDF-1 containing the normal gene and being suitable for autologous transplantation to correct the patient's genetic disorder.

47. A method according to claim 46, wherein the hematopoietic cells are derived from the patient's bone marrow or mobilized peripheral blood.

48. A method of autologous transplantation of immature hematopoietic stem cells for gene transfer to correct a patient's genetic disorder, which comprises:

(i) conditioning the patient under sublethal, lethal or supralethal conditions; and

(ii) transplanting the conditioned patient with a cell composition obtained according to the method of claim 46 or 47.

For the Applicants

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Patent Attorney

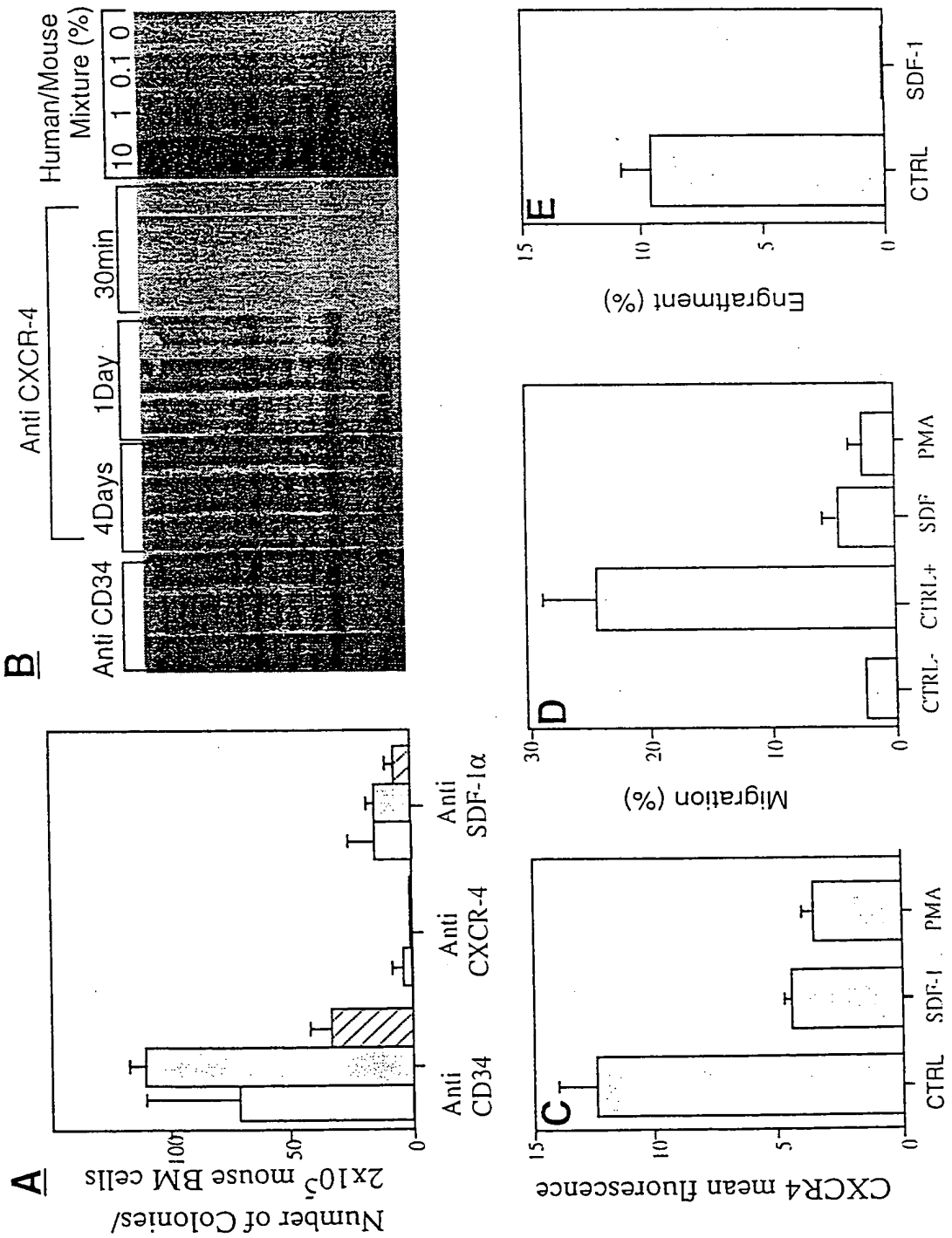
A
Number of Colonies/ 2x10^5 mouse BM cells
100
50
0
Anti CD34
Anti CXCR-4
Anti SDF-1α
B
Anti CD34
4Days
Anti CXCR-4
1Day
30min
Human/Mouse Mixture (%)
10 1 0.1 0
C
CXCR4 mean fluorescence
15
10
5
0
CTRL
SDF-1
PMA
D
Migration (%)
30
20
10
0
CTRL-
CTRL+
SDF
PMA
E
Engraftment (%)
15
10
5
0
CTRL
SDF-1

Figure 1

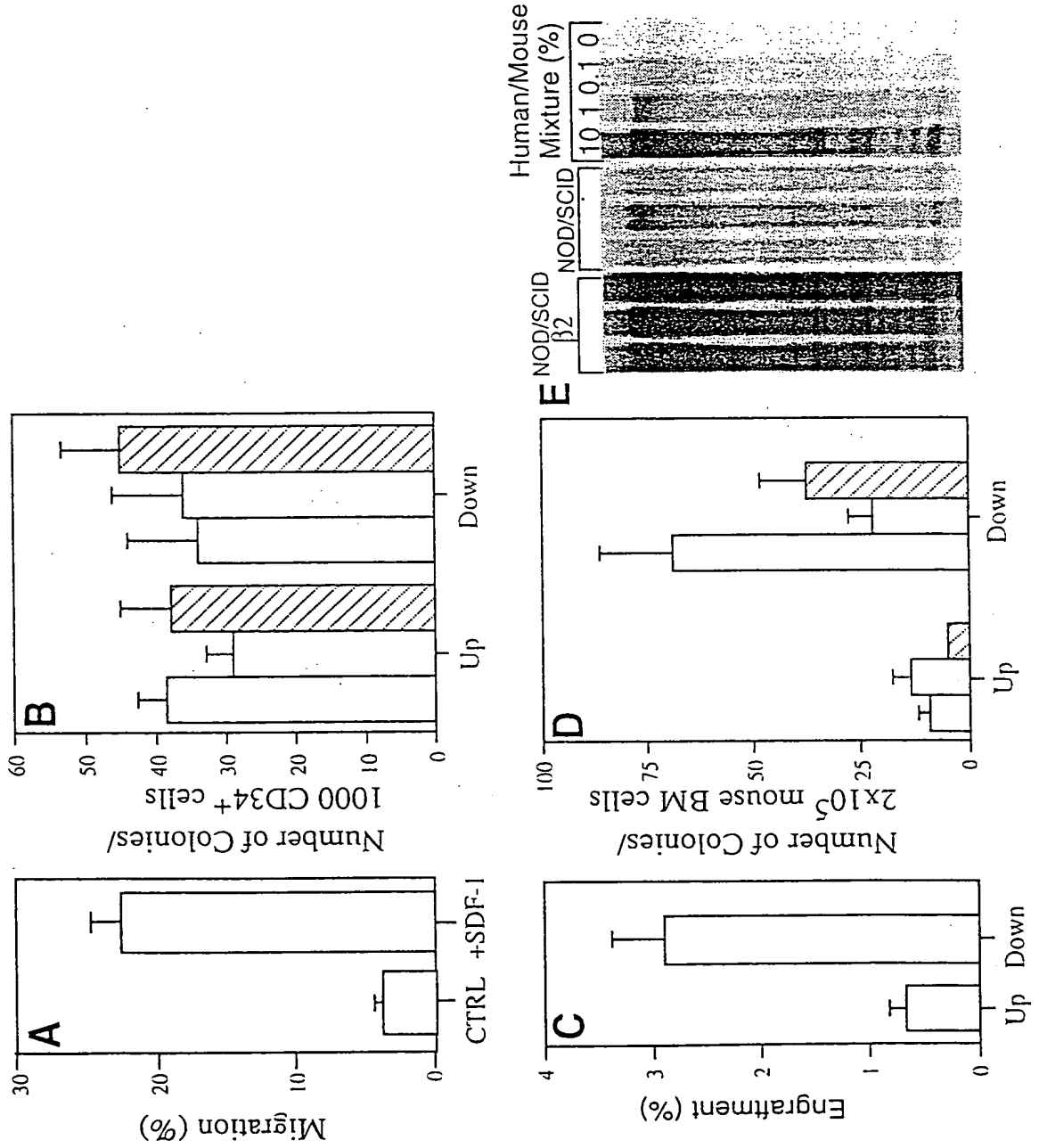
A
Migration (%)
CTRL +SDF-1
B
Number of Colonies/
1000 CD34+ cells
Up
Down
C
Engraftment (%)
Up Down
D
Number of Colonies/
2x10^5 mouse BM cells
Up
Down
E
NOD/SCID β2
NOD/SCID
Human/Mouse Mixture (%)
10 1 0.1 0

Figure 2

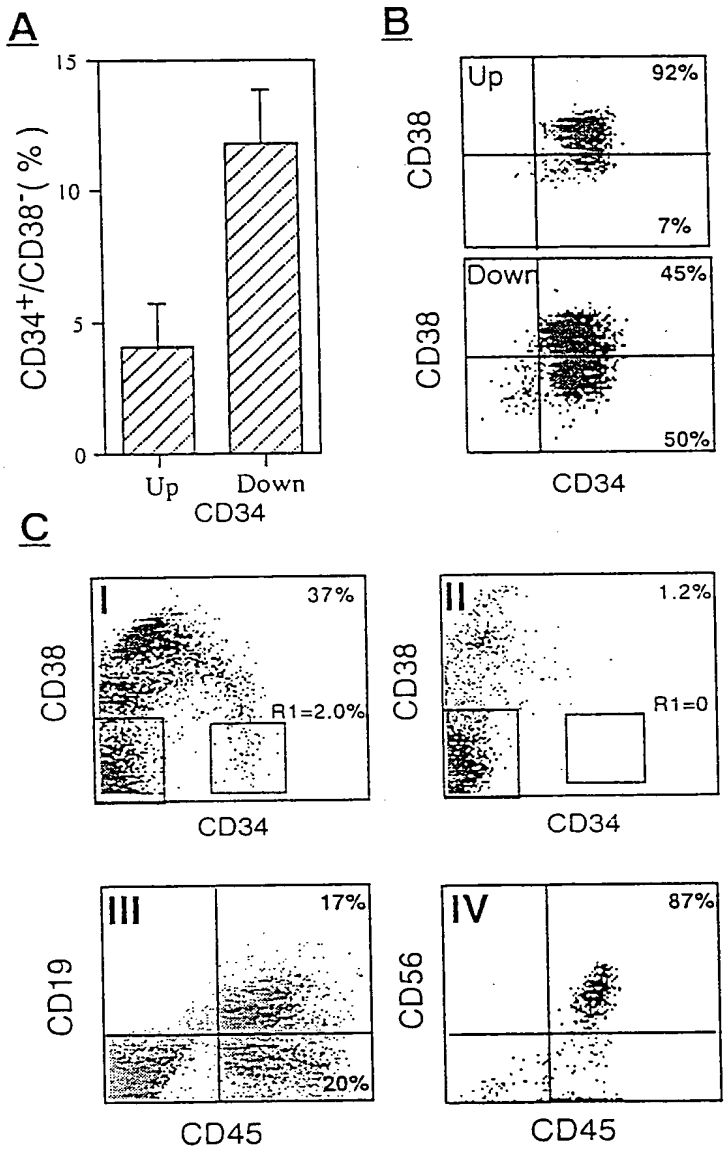
A
15
10
5
0
CD34+/CD38-(%)
Up
Down
CD34
B
Up
92%
7%
CD38
Down
45%
50%
CD38
CD34
C
I
37%
R1=2.0%
CD38
CD34
II
1.2%
R1=0
CD38
CD34
III
17%
20%
CD19
CD45
IV
87%
CD56
CD45

Figure 3

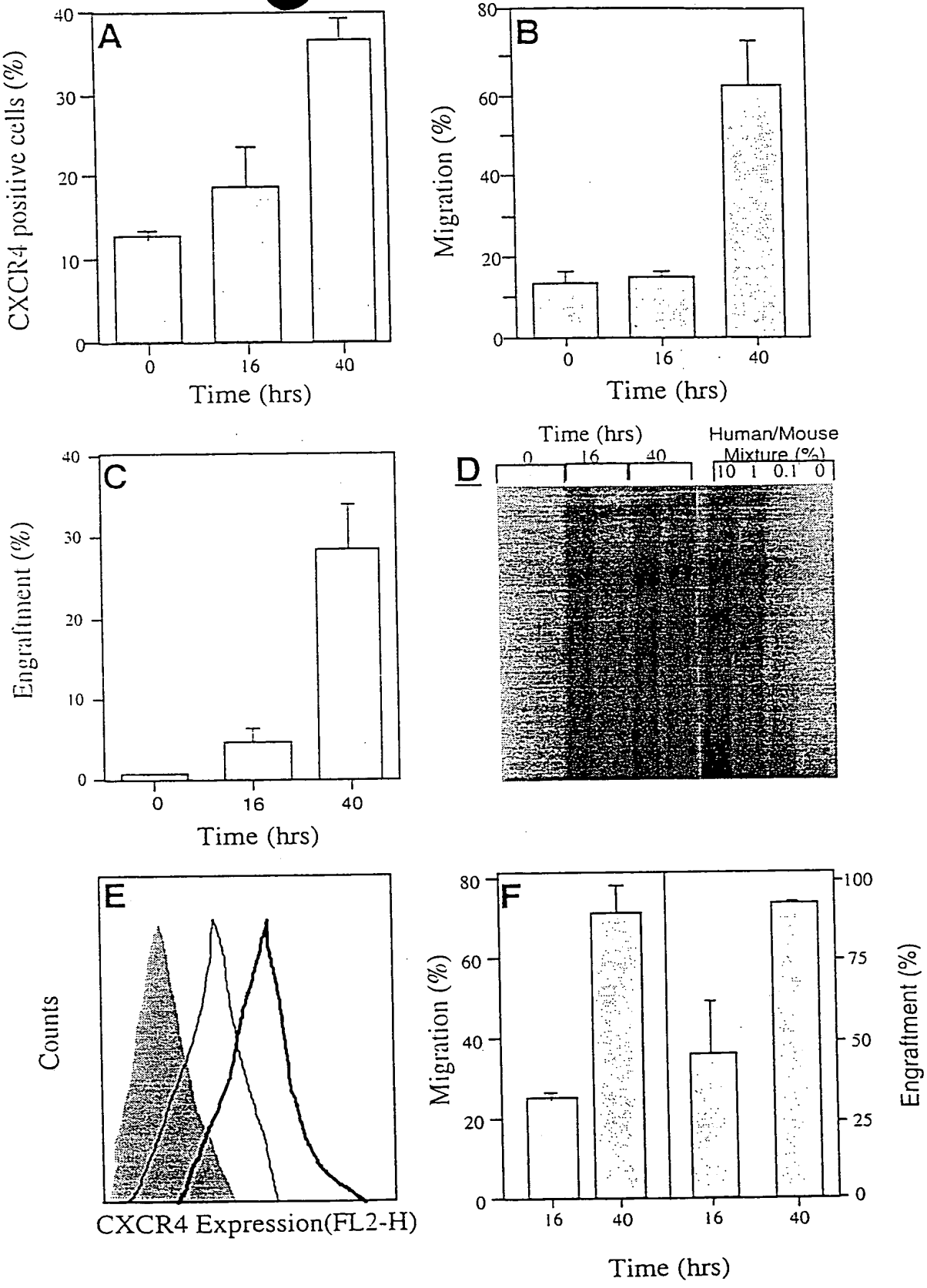
A
CXCR4 positive cells (%)
Time (hrs)
B
Migration (%)
Time (hrs)
C
Engraftment (%)
Time (hrs)
D
Time (hrs)
Human/Mouse Mixture (%)
E
Counts
CXCR4 Expression(FL2-H)
F
Migration (%)
Engraftment (%)
Time (hrs)

Figure 4

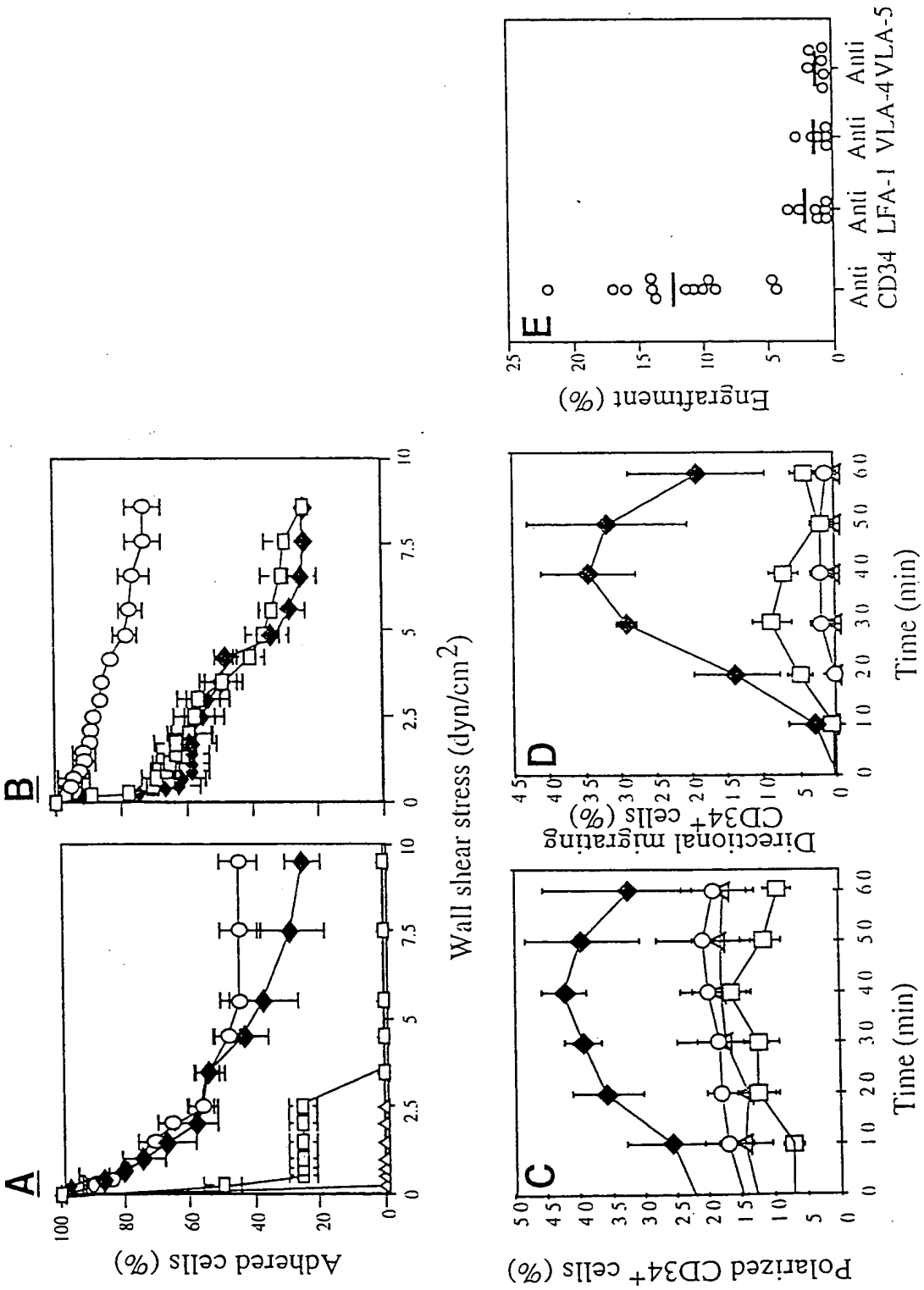
A
B
C
D
E
Adhered cells (%)
Wall shear stress (dyn/cm2)
Polarized CD34+ cells (%)
Directional migrating CD34+ cells (%)
Time (min)
Time (min)
Engraftment (%)
Anti CD34
Anti LFA-1
Anti VLA-4
Anti VLA-5

Figure 5

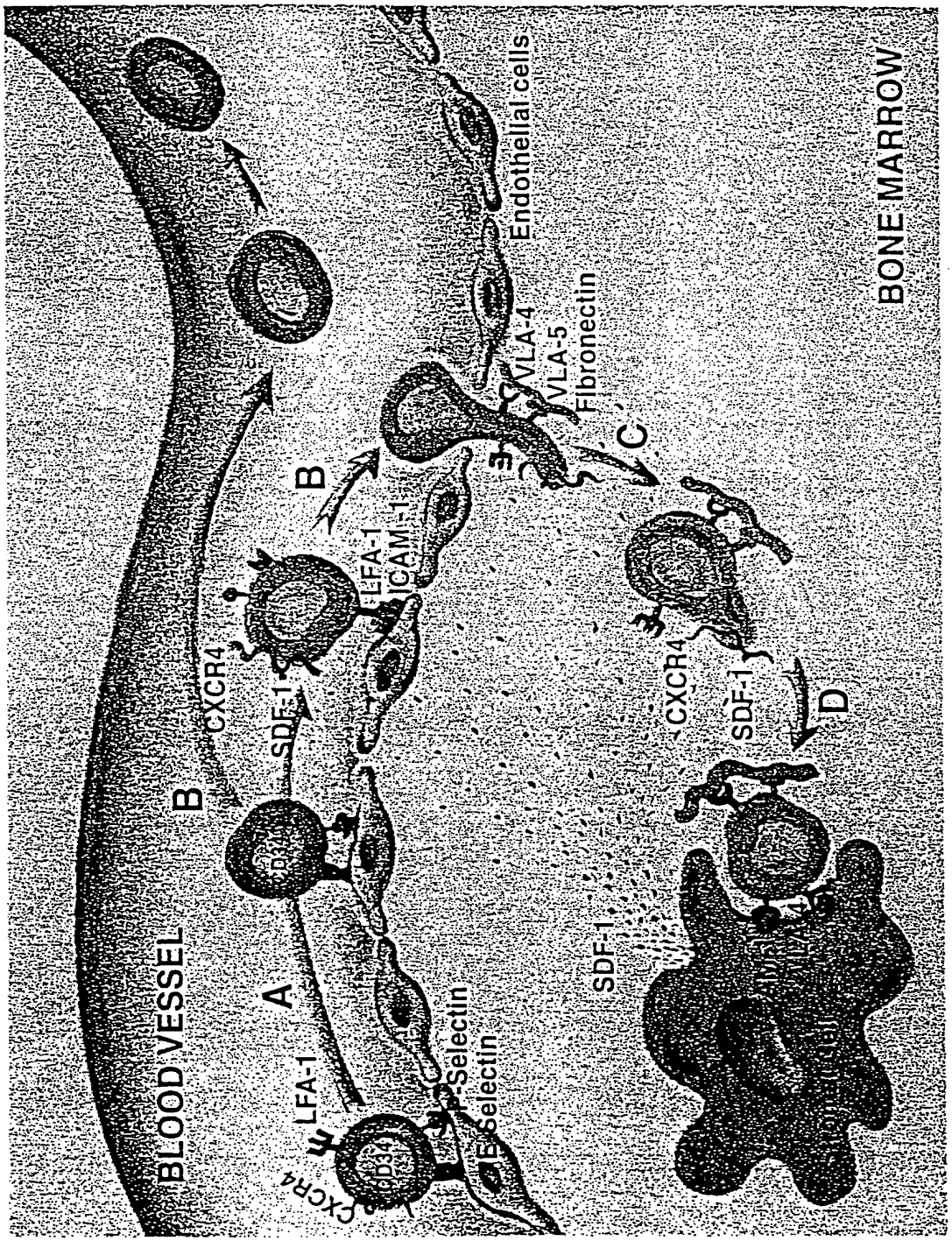
BLOOD VESSEL
BONE MARROW
Endothelial cells
A
B
B
C
D
CXCR4
SDF-1
LFA-1
ICAM-1
VLA-4
VLA-5
Fibronectin
P-Selectin
E-Selectin
CD34

Figure 6